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THE ROLE OF COAGULATION IN THE FORMATION OF PERITONEAL ADHESIONS

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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by
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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE ROLE OF COAGULATION IN THE FORMATION OF PERITONEAL ADHESIONS", submitted by C. van Breemen, D.V.M., in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Four groups of rabbits were injected once intraperitoneally with sterile talcum powder. These groups received respectively series of peritoneal infusions with AFIF, heparin, fibrinogen and albumin, lasting for five days. The rabbits receiving albumin and those receiving fibrinogen showed extensive peritoneal adhesion formation, whereas those receiving AFIF showed none or very few adhesions. The rabbits receiving heparin died early because of hemorrhage.

At the end of the five day experimental period none of the rabbits showed significant deviations in platelet and leukocyte counts. The groups receiving albumin and AFIF had low hematocrit and hemoglobin values and very high plasma fibrinogen concentrations.

Single intravenous injections of albumin and AFIF were also found to cause a significant rise in plasma fibrinogen concentration.

By in vitro experiments it was shown that such a rise in plasma fibrinogen concentration reduced the anticoagulant effect of AFIF.

TABLE OF CONTENTS

Introduction.. 1

Part 1: Peritoneal Adhesions.......................... 15

Materials and Methods................................. 15

Tests... 20

Adsorption of AFIF from Peritoneal Cavity. 27

Effects of AFIF on Peritoneal Adhesion Formation...................................... 29

Effects of Intraperitoneal Injections of Fibrinogen on Adhesion Formation....... 39

The Effects of Intraperitoneal Heparin Injections with the Presence of Talc in the Peritoneal Cavity................. 40

Part 2: Effect of Parenteral AFIF and Albumin on Fibrinogen Concentrations............. 41

Anticoagulant Activity of Intraperitoneal AFIF at the Beginning and End of a Five Day Period of AFIF Administration......... 41

An Attempt to Maintain in Vivo Incoagulability of Rabbit Blood by Repeated Intravenous Injections of AFIF......................... 42

The Effect of a Single Dose of AFIF on AFIF Titration, Thrombin Time, and Plasma Fibrinogen Level.......................... 44

The Effect of a Single Dose of Albumin on AFIF Titration and the Plasma Fibrinogen Level.................................. 49

The Effect of Single Saline Injections on the AFIF Titrations and the Plasma Fibrinogen Levels......................... 50

Discussion.. 52

Bibliography.. 60

TABLE OF TABLES

Table 1........... Peritoneal absorption of AFIF

Table 2........... Values for adhesion experiments

Table 3........... The haematological data

Table 4........... Effect of I.V. AFIF

Table 5........... Effects of increased fibrinogen concentration

Table 6........... Effects of increased fibrinogen concentration

Table 7........... Effects of I.V. albumin

Table 8........... Effects of I.V. saline

TABLE OF FIGURES

Figure 1........... Peritoneum of albumin treated rabbits

Figure 2........... Peritoneum of AFIF treated rabbits

Figure 3........... Photomicrograph of an adhesion (L.P.)

Figure 4........... Photomicrograph of an adhesion (H.P.)

Figure 5........... Peritoneum of fibrinogen treated rabbit

Figure 6........... Peritoneum of heparin treated rabbit

INTRODUCTION

As fibrin formation is the last step in the coagulation of blood its importance in haemostasis has been repeatedly stressed. This emphasis on the haemostatic role of the clotting of fibrinogen, may have contributed to the relative lack of interest in other functions of fibrin formation, which appear just as essential to the well being of an animal as the stoppage of blood flow from an injury.

The purpose of this thesis is the study of one of the non haemostatic functions of fibrin, namely its role in the formation of peritoneal adhesions. It seems therefore appropriate to begin this paper with a brief review of some of the most important functions of fibrin not directly related to haemostasis.

Intravascular fibrin deposition

It has been suggested recently that under normal circumstances intra vascular clotting proceeds continuously, but extremely slowly.(1) In 1956 Astrup postulated that such normal intra vascular fibrin deposition was due to the release of small amounts of tissue thromboplastin from very minor injuries due to the stresses of ordinary daily living. The fibrin so formed is thought to be removed by the lytic action of plasmin . (2) Very

convincing evidence has been put forward to show that physiologic intra vascular fibrin deposition occurs. The turnover time has been estimated for a number of clotting factors, and was found to be much faster than would be expected from their in vitro stability. Levene scraped fine layers off the intima of normal aortas. When he examined these with the electronmicroscope he found that the intima was covered with a thin layer of fibers, which showed a characteristic periodicity of 230 angstrom units, suggesting it to be fibrin.(3)

The physiological role of a thin coating of fibrin on the vascular wall might be to provide a smooth surface for the blood flow, thus reducing friction, and protecting the intima from the friction layer of the flow. For many years Duguid has put forward the hypothesis that atherosclerosis, as we know it in man, is largely the product of thrombosis.(4) It is beyond the scope of this introduction to evaluate the causes of atherosclerosis but there is a great deal of evidence suggesting it to be a pathological manifestation of the normal mechanism of intimal lining with fibrin.

Intra vascular fibrin deposition plays an essential part in metastasis of cancer. Although this role is pathologic rather than physiologic, it deserves brief

mention in this context as it is a further illustration of the relationship between fibrin formation and tumor growth. Wood(5) has followed the course of metastasis in vivo by injecting a suspension of V2 carcinoma cells into the central artery of a rabbit ear and observing microscopically the vascular bed of a previously installed ear chamber. Cancer cells were seen to lodge in the capillaries by sticking to the epithelium. After a few minutes the formation of a thrombus around these cells became apparent. Within a period varying from 30 minutes to several hours, a progressive loss of endothelial definition occurred in the area immediately adjacent to the cancer cells and the thrombus. Leucocytes then migrated through the injured epithelium causing defects through which cancer cells migrated into the perivascular connective tissue. Due to the fact that in histologic sections of the earliest stages in the development of metastasis, [illegible] tumor cells are frequently observed in intravascular thrombi, and from the findings that anticoagulants reduce the number of metastatic tumors, Wood concluded that the fixation of cancer cells in [illegible] intravascular thrombi is a factor of importance in the development of metastasis from blood borne cancer cells. Fisher and Fisher (6) confirmed the [illegible] by testing the effect of the anticoagulants [illegible] the implantation of

thrombus formation rather than through inhibition of the initial lodgement of the cancer cells. They injected a definite number of Walker carcinoma cells into the portal veins of heparinized and unheparinized rats and almost simultaneously aspirated a sample from the hepatic vein. No difference in the number of cells that passed through the liver could be found, indicating that the incidence of lodgment was the same for both groups. Fisher and Fisher also convincingly showed that heparin and fibrinolysin significantly reduced the incidence of metastasis.

Fibrin formation and its protective action against infection

When an irritant is injected into a body tissue, it will stimulate an inflammatory reaction, which can be recognized as defense reaction on the part of the living animals. Inflammation is the vascular, lymphatic and local tissue reaction which tends to localize and ultimately destroy or discard the injurious agent. At present no doubt exists that the clotting of fibrin is the first localizing factor, preventing the spread of the irritant.

The tissue damage causes a great increase of capillary permeability through the action of a permeability factor. Such a permeability factor is formed in the first stages of coagulation by the activation of a globulin by Hageman factor.(7)

The resulting inflammatory exudate carries with it into the damaged tissues prothrombin and fibrinogen. The prothrombin of the exudate is converted to thrombin by the tissue thromboplastin which is released from the damaged tissue cells. The thrombin then splits off fibrinopeptides from the fibrinogen molecules which now polymerize into fibrin strands which connect to form an extensive fibrin network in the damaged area, and in the lymphatics leading away from the area. The fibrin network so formed will retard the bacterial spread by a reduced filtration and probably also through adsorption onto the immense adsorbing area of the fibrin network.

Menkin (8) has done convincing experiments to showthat the clotting of the inflammatory exudate inhibits bacterial invasion. In one such experiment he injected an irritant into the peritoneal cavity and at different intervals of time thereafter, bacteria were introduced into the peritoneum. To test the spread of the bacteria he cultured the retrosternal lymph nodes and counted the bacterial colonies so obtained. He found a much greater number of colonies on the cultures from the animals not injected with the irritant than on the cultures from the animals injected with the irritant prior to introduction of the bacteria. Menkin also studied the spread of dyes injected into connective tissue. When such a dye was installed into an inflamed

area it was seen to spread over a much shorter distance than when it was injected into healthy connective tissue. When dye was injected at the periphery of the inflamed area it did not penetrate such an area. Menkin in extensive histological studies of inflamed areas found that the lymphatics draining such areas were thrombosed.

Glenn, Peterson and Drinker (9) demonstrated that the tissue thromboplastin is the probable agent initiating lymphatic thrombosis. They introduced tissue extracts into burned areas and found this procedure greatly accelerated lymphatic thrombosis.

Day (10) postulated that besides lymphatic blockage the retarded filtration rate of fluid through an inflamed area is the result of formation of protofibrils or primary fibrin polymers in the matrix of the connective tissue. The supporting evidence for this theory comes from the following experiment. Day attached a piece of mouse subcutaneous connective tissue to the end of a narrow tube so as to form a diaphragm closing the tube. He then measured the rate of flow of citrated serum and plasma which was caused to flow through this diaphragm under a pressure of four centimeters of water. Then the flow was shut off and a large quantity of thrombin added to the plasma inside the tube, the plasma in the

tube and in the connective tissue clotted. This process slowed down the rate of filtration considerably.

When very small amounts of thrombin were added while the plasma was still flowing, a slow clotting process was in progress as the plasma filtered through the connective tissue. This procedure slowed down the rate of filtration twice as much as the sudden clotting of the plasma and the tissue fluid. Day considers connective tissue to consist essentially of a protein network arranged in layers and having meshes of macromolecular dimensions.(11) Slowing of flow was shown to take place when solutions of macromolecular substances such as starch or dextran were filtered through the tissue. This slowing was considered to be due to blockage of the meshes by macromolecules or macromolecular aggregates. Day assumed that in a similar way the particles formed during clotting became impacted causing a decreased permeability of the tissue.

Besides the filtration action of fibrin studied by Menkin, it is apparent that diminution of the flow of fluid through a zone of inflammation must lead to a restriction of the escape of tissue fluid and lymph into the surrounding normal tissue. In this way the spread of noxious material from an inflamed area is also limited. Thus more time is available for immune reactions and phagocytosis

to render the irritant innocuous.

Coagulation and bacterial invasiveness

Menkin has shown that bacterial invasiveness is directly related to the amount of fibrin deposited during the local inflammatory reaction. Bacteria introduced into normal tissue are likely to invade the tissue and enter the systemic circulation. Various bacteria vary in this capacity. Menkin made use of this difference in invasiveness between different species of bacteria to elucidate factors governing bacterial invasiveness. Three microbes were studied, namely: Staphylococcus aureus, pneumococcus type 1, and a strain of haemolytic streptococci. These organisms were injected into the skin of the fore limb of a rabbit. A diazo dye, trypan blue, was then injected into the area and the tributary lymph-nodes and lymphatics were examined histologically at successive intervals to see whether passage of the dye had been blocked by the process of local inflammation. When the local inflammation had been caused by Staphylococcus the lymphatics draining the area were thrombosed within one to four hours after the injection. The rapid clotting induced by staphylococci is partly due to its production of the procoagulant coagulase. In contrast, at nineteen hours after the introduction of streptococci the lymphatics

were still patent, although at this time the beginnig of dammage to the endothelial wall was perceived. Two days after the introduction of streptococci the lymphatics were finally blocked by thrombi. The pneumococci stimulated inflammation , which caused lymphatic thrombosis after six to nine hours. Other workers have shown the importance of the fibrin network in tissue spaces in reducing invasion by other species of bacteria. Lebowich, McKillip, and Conboy (12) demonstrated the primary mechanism responsible for localization of human cutaneous anthrax to be the mechanical barrier consisting of clotted exudate.

It is thus evident that virulence and invasiveness are different concepts. Virulence is the bacteriums capacity to dammage tissue. Since very virulent microbes stimulate excessive exudation and tissue thromboplastin release, fibrin formation is most effective in preventing the spread of the most harmful organisms.

Coagulation and connective tissue formation

It is obvious that fibrin formation precedes such processes of connective tissue formation as seen in the organization of a thrombus, the encapsulation of an irritant, wound healing, and adhesion formation. There is an accumulating body of evidence that fibrin formation

not only precedes these various forms of connective tissue formation, but that it is essential for the normal sequence of events. Biggs visualizes these events as a growth of granulation tissue into a mass of fibrin, which neatly fills the cavity to be obliterated or surrounds an irritant (13). This fibrin network would serve as a scaffolding to guide the fibroblasts and the new capillaries, and would be lysed after it had fulfilled this function. MacCallum (14) in 1940 ascribed two more functions to the fibrin network, namely a bactericidal and a nutritive one.

How many of these roles postulated for blood and exudate coagulation are fact, and how many are fiction? At the present there appears to be enough evidence to state that fibrin formation plays a role in connective tissue formation, and that in certain circumstances it is essential as will also be shown in this thesis.

What evidence is there for a dependence of connective tissue growth on fibrin formation?

Only recently has experimental work provided proof for a causal relationship between normal clottingand normal fibroblast growth, although clinical observations had suggested such a relationship before. Biggs (13) concludes from many case reports of haemorrhage persisting

for many days and weeks in haemophylic subjects who had received small cuts or abrasions, that wound healing is deficient because of this coagulation disorder. Fraenkel and Honey's observation (15), that large wounds in a haemophylic patient healed rapidly during the administration of animal antihaemophylic globulin, suggested that connective tissue formation can be due to the absence of a single clotting factor. Astrup (16) noted that patients with afibrinogenemia had less pathologic joint changes than haemophylic patients. He ascribed this difference to a failure of fibrin deposition in the cases of afibrinogenemia.

Although the above observations are suggestive they are either too sporadic or too inconsistant to constitute a proof. Recently a Swiss family has been discovered of which some of the members suffered from a haemorrhagic diasthesis which also caused abnormal wound healing. Duckert et al, in Zurich have reported on this disease. Their main findings will be described below because they definitely relate clotting with connective tissue formation. (17)

Seven members, which includes both males and females, of this family had a history of bleeding. All seven experienced umbilical bleeding twelve to fourteen days

after birth, which proved fatal for one of them. After very minor injury prolonged bleeding and haematoma formation would occur. Immediately following injury haemostasis in these patients was normal, but bleeding usually started again 24 to 36 hours later. Their wounds healed very slowly with the formation of excessive granulation tissue, and resulting in extensive, irregular and retracted scars. Standard coagulation tests showed both the intrinsic and the extrinsic coagulation mechanisms to be normal. The only abnormal finding was an abnormal thrombelastogram, which indicated that the firmness of the blood clot was less than normal and that its strength decreased rapidly. This decreased clot stability was shown to be due to the absence of fibrin stabilizing factor (FSF), also called fibrinase, from the blood of the patients.

Laki and Lorand in 1948 (18) were the first to describe this factor, which is essential for the formation of a normal clot. These workers demonstrated that fibrin obtained from the action of thrombin on well purified fibrinogen and calcium was soluble in 5 molar urea. Later Loewy et al., (20) and Lorand and Jacobson (19) showed in plasma that the inability of the fibrin clot to dissolve in 5 molar urea was due to the action of a globulin. This globulin was given the name 'fibrin stabilizing factor' by Lorand,

and fibrinase by Loewy and co-workers. (20)

In accordance with the above findings, Duckert et al. found the plasma clots of their patients to be soluble in 5 molar urea. In vitro and in vivo additions of very small amounts of normal blood would render the clots insoluble in urea. Normal blood also corrected the abnormal tissue growth. One patient was givena blood transfusion before the extraction of three molars. This procedure prevented excessive bleeding and also caused the wounds to heal rapidly with normal cicatrization.

Beck et al. made use of this clotting defect to show a dependence of organized growth of fibroblasts on normal clot formation. They grew subcultures of human heart fibroblasts in plasma clots from FSF deficient patients and in clots from normal individuals. The cultures in the normal plasma clots were abundant, very regular in shape and had elongated cells and the formation of collagen fibers. The cultures in the FSF deficient clots were scanty, there was loss of contact between the cells and their nuclei and cytoplasm were of irregular shape. In the latter cultures no collagen formation could be observed and the cells eventually became necrosed.

This group of workers postulated as one of the

reasons for the abnormal fibrous tissue growth, that in the absence of FSF the fibrin is not able to provide the growth promoting mechanical traction.

As the only function of AFIF presently known is inhibition of fibrin formation, it was decided to use it to prevent the connective tissue formation as seen in peritoneal adhesions.

Part 1. PERITONEAL ADHESIONS

Materials and methods

Anticoagulant Fraction of Incubated Fibrinogen (AFIF):

AFIF was prepared from Laki's fibrinogen (21) as described by Triantophyllopoulous (22).

Since the experiments reported here required large amounts of AFIF, a few minor alterations were made which speeded up the production. For this reason, the preparation as done for these experiments, will be described below.

Production of AFIF

1. Dissolve 7 gm. of Armour's fibrinogen fraction 1 in 350 ml. of phosphate buffer pH 6.4

Since 4 seven gm. lots were prepared each time, 1400 ml. of phosphate buffer were made up by mixing 1029 ml. monosodium phosphate (27.8 gm./litre) with 371 ml. of disodium phosphate (53.65 gm. $Na_2HPO_4.7H_2O$/litre or 71.7 gm. $Na_2HPO_4.12H_2O$/litre).

2. Dilute with equal amount of distilled water, which should bring the pH to 6.52

3. Place the 4-1000 ml. beakers in the refrigerator overnight.

4. Take the solutions out of the refrigerator and filter immediately.

5. Add 1/3 of the filtrate volume of saturated ammonium sulfate. This will almost fill the 1000 ml. beakers.

6. Leave the mixtures stand for at least one hour to allow the precipitated fibrinogen to rise to the top.

7. Take the fibrinogen floating on the surface off with a spatula and centrifuge this at 12000 RPM for five minutes.

8. Dissolve the precipitate in as small a quantity of normal saline as possible (about 300 ml.).

9. Dialize against 0.3 M KCl and change the dialysate until it does not produce any cloudiness with $BaCl_2$.

10. Filter and determine the protein content by doing a biuret test.

Biuret test: Add 0.5 ml. of the fibrinogen to a cuvette. Add 1.5 ml. of 0.3 M KCl to this , and 2 ml. KCl to another cuvette. Add 4 ml. Biuret reagent to each of the two tubes. Place the blank in the Junior spectrophotometer and set at 100. Take a reading on the tube containing the fibrinogen and read the protein concentration from a curve relating light intensity to concentration.

11. Dilute the fibrinogen solution to about 11 mg./ml. and add 10% imidazole buffer.

12. Add 1/5 of a bottle of streptokinase activated human fibrinolysin (donated by Ortho Canada) to each gm. of fibrinogen in solution. This amount is not definite, but has to be adjusted so that the fibrinogen will be sufficiently lysed in three days or slightly longer.

13. Seitz filter this mixture and incubate at 37 C.

14. Every day a 1 ml. sample is taken out under sterile conditions and some thrombin is added. When the sample remains clear or becomes faintly cloudy, the solution is taken out of the incubator.

15. Add Epsilon-amino-caproic acid to a concentration of 0.1 M.

16. Add 0.33 of this volume saturated ammonium sulfate.

17. Place in the ultra-centrifuge and turn it on full speed. As soon as the speed is 10,000 RPM, switch the centrifuge off. Save the supernatant and refill the centrifuge tubes leaving the precipitate at the bottom.

18. Add 0.5 of the supernatant volume saturated ammonium sulfate to the supernatant. Centrifuge above, but this time discard the supernatant.

19. Dissolve the precipitate in as little normal saline as possible.

20. Dialyze against normal saline until no more precipitate forms on the addition of $BaCl_2$.

21. It is possible to obtain a concentration of 35 mg./ml. of AFIF solution by this procedure. If however, the concentration is too low, it may be doubled by dialyzing it against 0.45% saline, then hanging the dialysis bag in a

refrigerator for a couple of days until halfof the volume has evaporated off. This is followed by one dialysis against 0.9% saline.

22. Epsilon-amino-caproic acid is then added to a level of 50 mM./liter.

23. This solution is then Seitz filtered and stored in the refrigerator until used.

Albumin: Bovine albumin fraction V was dissolved in 0.9% saline to the same concentration as the AFIF used for peritoneal injections. EACA was added to reach a concentration of 50 mM., and the solution was Seitz filtered and kept refrigerated.

Fibrinogen: Laki's fibrinogen prepared from Armour's Bovine Fibrinogen Fraction 1 was prepared as described in the first 10 steps of the AFIF preparation. The solution was then dialyzed against normal saline, after which it was Seitz filtered and stored under refrigeration. Due to precipitation of fibrinogen under high concentrations, the fibrinogen was used for intraperitoneal injections had a concentration of 19 mg./ml.

Heparin: Lipo-Hepin (Riker Pharmac. Co. Ltd., Cooksville, Ontario) was diluted to two mg. per ml., Seitz filtered and kept under refrigeration.

Talcum Powder: Three gms. of talcum powder (Talc No.7 The British Drug House, Canada Ltd., Toronto) were suspended in eight mls. of normal saline and autoclaved.

Penicillin-Streptomycin: Aqueous suspension of Penicillin Hydro-chloride 200,000 units/ml. and dihydro-streptomycin sulfate 0.25 gm./ml. (Stevenson, Turner & Boyce, Guelph, Ontario)

TESTS

Hematocrit:

Aheparinized capillary tube (Schieffelin & Co., New York) was filled three-quarters full with fresh blood. One end of the tube was sealed with a flame. The tube was then spun at 3000 RPM for 15 minutes and the portion of the total volume occupied by the red blood cells was read off with theaid of a millimeter ruler.

Hemoglobin:

Principle: Ferricyanide converts hemoglobin from the ferrous

to the ferric state to form methemoglobin, which then combines with the K-cyanide to produce the stable pigment cyanmethemoglobin (24). These two reactions are rapid and stoichiometric, so that the color intensity is proportional to the concentration of the hemoglobin.

Reagents: Drabkin's diluent solution consists of: $NaHCO_3$ 1.0 gm., potassium cyanide (KCN) 50 gm.,potassium ferricyanide ($K_3Fe(CN)_6$) 200 mg., and distelled water to one liter. This is a clear solution pale yellow in color.

Procedure: An accurately measured volume of blood (0.02ml.) was diluted in 5 ml. of Drabkin's solution. The optical density of this solution was then compared with that of the standards. The optical density is taken to be directly proportional to the concentration of the pigment. The color intensity was measured in a junior spectrophotometer set at 540 mµ.

White blood cell count:

Using a special diluting pipette the citrated blood was diluted 1 to 20 with 3% acetic acid (24). The acetic acid haemolyzes the red cells and thus makes the leukocytes easier to count. The pipette was shaken

for about half a minute. A cover glass was placed on the Neubauer counting chamber. Several drops of fluid were discarded from the pipette to remove the fluid in the capillary portion of the pipette, which was not mixed with the blood. A small quantity of the diluted blood was then placed between the cover glass and the ruled platform of the counting chamber. The fluid will run underneath the cover glass by capillary attraction. The count is made in five large squares and the number of cells found in these five squares is multiplied by 40 to give the count per cubic mm, The average of two such counts was used for the data presented.

Platelet count:

Citrated blood was drawn up to the 0.5 mark of the red cell count diluting pipette and filled to the 101 mark with diluting fluid (i. e. sodium citrate 3.8 gm, formaldehyde 0.2 ml, distilled water 100 ml. This diluent was filtered before use (25).) The pipette was shaken for two minutes, a few drops discarded and a small amount placed in the counting chamber. The platelets were allowed to settle by placing the counting chamber in a Petri dish for 15 minutes. To prevent drying a small sponge soaked with water was also placed in the

dish. The platelets were counted under high power in five small squares of the Neubauer counting chamber. The number of platelets thus found was multiplied by 10,000 to give the platelet count per cubic mm. The average of two such counts was used for the data presented here.

Thrombin clotting time:

Two tenth of a ml. of citrated plasma were measured into a test tube. After warming it in a water bath at 37 degrees for 15 seconds, 0.1 ml. of thrombin solution was blown into the same tube, and the time required for a clot to form was carefully determined by means of a stopwatch. The thrombin solution employed was a 100 fold dilution in distilled water of Thrombin Topical, Parke Davis & Co. Ltd., and contained 10 NIH units per ml.

Fibrinogen determination:

Principle: The plasma is clotted by adding excess calcium chloride to a mixture of 25 ml. saline and 1 ml. oxalated plasma. The amount of fibrin clotted is determined from its tyrosine content (25).

Reagents: Calcium chloride 1M.
Sodium hydroxide 10%

Sodium carbonate 20 per cent.

Phenol reagent 2N Fisher Scientific Co..

Procedure: One ml of oxalated plasma was diluted with 25 ml distilled water in a 30 ml beaker, and 0.2 1 M. $CaCl_2$ were added and mixed. After 30 minutes the clot was removed by wrapping it around a stirring rod. It was then washed with distilled water and put in a test tube. The clot was digested by covering it with 0.5 ml of 10 per cent NaOH and heating it for 30 minutes in a boiling water bath. The alkaline solution was diluted in a volumetric flask to 25 ml with distilled water. Five ml of this solution were transferred into a large cuvette and 4.5 ml distilled water added. Nine and a half ml distilled water were added to another cuvette , which served as a blank. To each of these tubes 0.5 ml of Phenol reagent and 3 ml of 20 per cent sodium carbonate were added. After mixing, the reaction mixture was allowed to stand for 30 minutes to allow maximum color development. After this time the light transmittance of the samples was read using a Coleman Junior Spectrophotometer set at a wave length of 560 mu. The transmittance was converted into mgs of tyrosine using for this purpose a tyrosine calibration curve.

Fibrinogen determinations were also done using

normal saline for diluting the plasma samples. Both the determinations in saline and distilled water were done in duplicates.

The tyrosine calibration curve was constructed as follows: 0.02, 0.04, 0.06, 0.08, and 0.1 mg of tyrosine contained in a series of cuvettes were diluted to 9.5 ml with distilled water. Into each of the tubes 0.5 ml of phenol reagent and 3 ml of 20 per cent sodium carbonate were added. After 30 minutes the intensity of the color was read with the C.J.S. set at a wavelength of 560 mu. From the values obtained a curve was constructed on semi-logarithmic graph paper.

To obtain the quantity of fibrinogen in mg per 100 ml of plasma the tyrosine value was multiplied by the conversion factor 5350.

Titration of AFIF with fresh blood: (A method to determine AFIF activity)

To a number of 4 ml test tubes, which are marked at 2 ml, the following quantities of the AFIF to be tested were added: 0.18, 0.2, 0.22, 0.25, 0.29, 0.33, 0.4, 0.5, and 0.67 ml . To each of these, fresh rabbit blood was added to give a total volume of 2 ml for each tube. The tubes were than covered with Parafilm and their content

mixed by inverting three times. The mixture with the lowest ratio of AFIF/blood which did not clot was then taken to be a measure of the effectiveness of the AFIF solution tested. Depending on which tube this happened to be, the result of the titration could be expressed as 1/10, 1/9, 1/8, 1/7, 1/6, 1/5, 1/4, 1/3, and 1/2.

Absorption of AFIF from the Peritoneal Cavity

Experimental procedure:

Five rabbits were used for this experiment and were prepared as follows. Intravenous nembutal, 60 mg per ml was administered into the marginal ear vein. A calculated dose of 27 mg per kg of body weight was administered slowly, but mostly an additional 5 to 10 mg per kg body weight had to be given for proper surgical anaesthesia. The fur of the neck and the front of the chest was then clipped with animal clippers. A skin incision was made along the ventral surface of the neck and the external jugular vein exposed and catheterized with a 15 gauge polyethylene tube. A very small incision was then made through the linea alba just below the umbilicus, into the peritoneal cavity. The AFIF solution was injected through this opening, which was then closed by applying towel forceps.

The dose of AFIF was calculated as follows: The blood volume of the rabbit was taken to be 70 ml per kg of body weight. A titration of the AFIF solution with the blood of a donor rabbit was done. The dose of AFIF administered was twice the amount that would render incoagulable a volume of rabbit blood equal to the calculated

blood volume of the experimental rabbit. This was approximately 650 mg. per kg. of body weight.

At hourly intervals after the injection of AFIF, blood samples were withdrawn from the catheterand placed in a water bath at 37 degrees Centigrade. After half an hour the samples were examined for the presence of clotting. If clotting had taken place, the serum was diluted with 10mM. calcium chloride solution. If clotting was incomplete a clot would form upon such dilution.

Results:

Table 1 Peritoneal absorption of AFIF

Time	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5
1 hr.	clot	clot	clot	clot	clot
2 hr.	clot	no clot	no clot	no clot	clot
3 hr.	clot	no clot	no clot	no clot	clot
4 hr.	no clot	no clot	no clot	no clot	clot
5 hr.	clot	no clot	no clot	no clot	clot
6 hr.	clot	clot	clot	clot	clot

All serum samples tested showed fibrin formation. Rabbits one and five showed the most fibrin formation near the middle of the seven hour period.

The effect of AFIF on peritoneal adhesion formation

Surgical preparation of the rabbits:

In each experiment two rabbits were used, one receiving AFIF and the control receiving albumin. Both animals underwent the same surgical procedure.

The rabbits were anaesthetized with intravenous nembutal as described above. The left side and the back of the animal were clipped. This area was then first swabbed with ether followed by a zepherin chloride swab. A sterile technique was used throughout. A small incision was made through the skin of the left flank just behind the last rib. A similar incision was made through the skin of the back between the shoulder blades. Fine forceps and sharp pointed scissors were used to dissect through the abdominal muscles. Next the peritoneum was entered with the sharp scissors. The end of a soft catheter* was inserted into the peritoneal cavity. The other end was clamped in a haemostat and pushed from the flank incision through the subcutaneous tissue to the incision on the back, so that the catheter emerged from the skin at the base of the neck. The peritoneum was closed by

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* Infant feeding tubes - C.R. Bard, Inc., Summit, New Jersey.

simple continuous suture taking both theperitoneum and the abdominal muscles at once. The catheter was fixed in place by a stitch around a small piece of polyethylene tubing, which was molten crosswise onto the catheter. The skin was closed by simple interrupted sutures.

Even though the catheter protruded from the base of the neck, it appeared possible for the rabbits to pull it out, so that it was necessary to keep the rabbits in a rabbit box, or have a wide collar around their necks.

Cardiac puncture in a rabbit:

Though not strictly speaking a surgical procedure, the technique used here will be described as it was easy and quick and caused only one death due to haemopericardium out of at least 100 punctures.

The assistant held the unanaesthetized rabbit on its back by holding its hind legs in the right hand and the front legs and ears in the left hand. The operator stood on the opposite side of the table and faced the left side of the rabbit, which was stretched on its back. The left thumb is used to locate the rabbits left intercostal space where the pulsation of the heart is felt most markedly, while the left index finger locates a similar place on the right side of the thorax about one inch lateral to the sternum. The point of insertion

of the needle is the intercostal space located by the thumb one half inch from the midline. The direction of insertion is toward the tip of the operators index finger, and the needle usually needs to be inserted for half an inch or less to be able to withdraw blood.

Experimental procedure:

Thirty minutes after surgery, while the rabbit was still under anaesthesia, the first dose of AFIF or albumin was injected through the catheter into the peritoneal cavity. Three hours after the first dose a sterile suspension of talcum powder was injected into the catheter and flushed with 2 ml sterile saline. In the first four experiments a series of six-hourly intraperitoneal injections of AFIF in the case of the experimental animals and albumin in the case of the controls were started six and a half hours after surgery. In the last four experiments the multiple injections were replaced by a continuous infusion by an automatic slow injection apparatus *. The amounts of AFIF and albumin administered during the experimental period are given in table 2 as the dosage per six hours. A period of five days was allowed for adhesion formation except in the first two experiments, which

* Manufactured by C.F. Palmer, London , England, Ltd. .

lasted Six days.

As the rabbits used were crossbred domesticated animals and therefore most likely not free of disease, each one of them received one half ml. penicillin streptomycin per day during the experiment, to prevent flareups of chronic infections, which could result from diminished fibrin formation, or experimental stress.

Upon termination of the experimental period and in the case of the first four experiments three hours after the last injection, a blood sample was taken by cardiac puncture, part of which was oxalated and part citrated. The animal was then killed with an over-dose of nembutal.

The autopsy consisted of a gross examination of the peritoneal cavity and thoracic viscera, and a microscopic examination of some adhesions and peritoneal smears.

The blood samples were used for leukocyte and platelet counts, and haematocrit and haemoglobin and fibrinogen determinations.

Table 2

Values for adhesion experiments

No.	Dose mg/6hrs.	Adh.	W.B.C.	Pl.	Ht.	Hb.	Fb..	Th time seconds
C1		M						
E1	500	-						
C2	550	M			33		270 267	10.0
E2	550	-	6500	584000	27		1090 1020	27.7
C3	550	M	11720	700000	27	7.9	332 320	9.0
E3	550	F	10520	510000	30	9.2	722 540	30.0
C4	700	M	5320	620000	25	6.0	411 375	7.0
E4	700	F	3200	650000	25	6.4	756 575	11.5
C5	700	M	5160	360000	32	8.8	590 573	6.0
E5	700	-	3880	450000	28	8.3	847 738	16.0
C6	700	M	5400	560000	32	8.6	895 831	10.0
E6	700	-	3000	450000	29	8.1	875 690	20.0
C7	700	M	7880	600000	29	8.2	775 740	7.5
E7	700	-	5500	430000	27	8.0	730 515	30.0
C8	700	M	4360	690000	33	10.0	810 744	7.0
E8	700	F	4840	580000	32	8.8	1011 888	21.0

Results:

Table 2 is a summary of the data obtained.

The controls are indicated by C, and the experimental animals by E.

M indicates many adhesions, F indicates the presence of a few adhesions between the liver and the diaphragm, and '-' indicates the absence of any adhesions.

Ht. is the volume of packed red cells per 100 ml blood.

Hb. is haemoglobin in grams per 100 ml blood.

Fb. is the fibrinogen concentration in mgs per 100 ml. of plasma. The top figures for each animal indicate the concentrations obtained by clotting the plasma in distilled water and $CaCl_2$, and the second value was obtained by clotting the plasma in 0.85 % saline and $CaCl_2$.

The fibrinogen concentrations obtained with distilled water were higher than those obtained with saline. This difference was greatest in the animals receiving AFIF.

Th. time indicates the thrombin time.

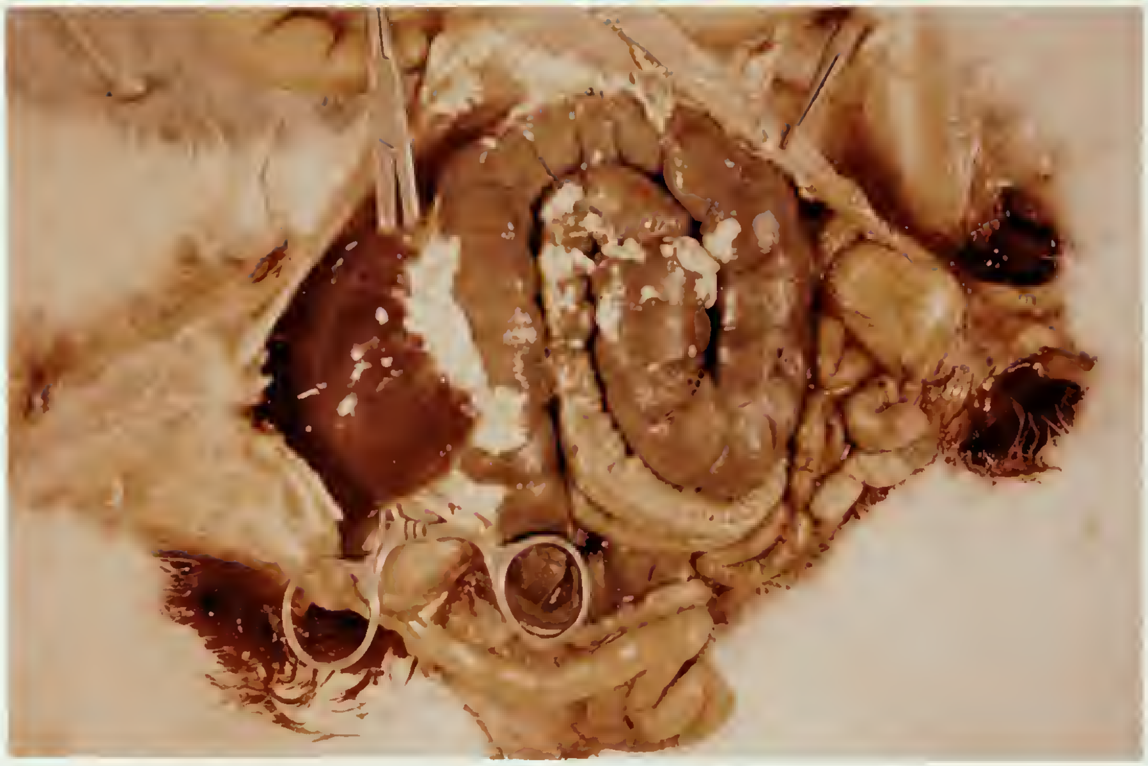


Figure 1

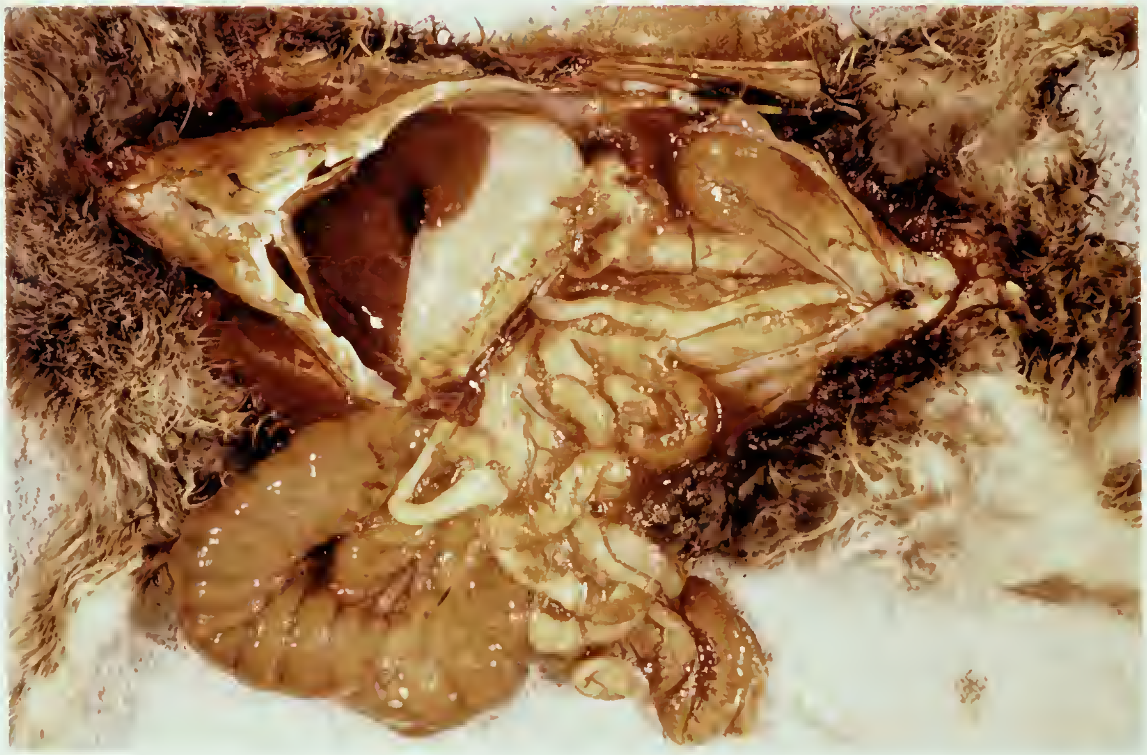


Figure 2

Gross appearance of the peritoneum.

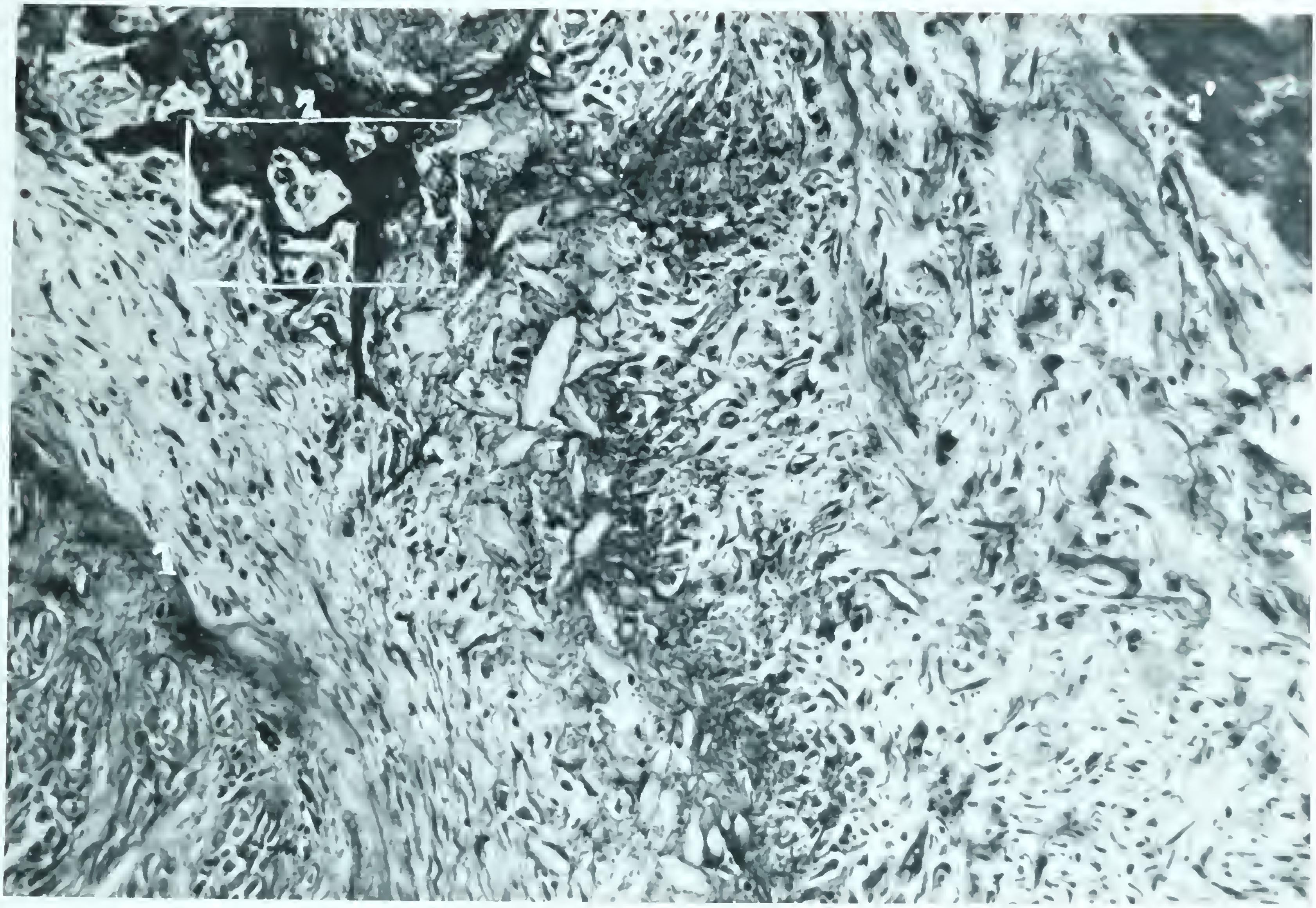
Figure 1 is a photograph of a control animal, which had received albumin. Massive adhesions could be seen where ever talc was in contact with two peritoneal surfaces as seen here between the liver and large intestine, and between the large intestine and the parietal peritoneum. Such adhesions were seen in all controls.

Another finding characteristic of the controls was that the talc was localized in large lumps, and where no talc was present, the peritoneum had a healthy shiny appearance. No cloudy exudate could be seen.

Figure 2 is a photograph of an experimental animal, which had received AFIF. Four animals had no adhesions, one of which is shown here, and four had a few adhesions between the liver and diaphragm. All experimental animals showed signs of inflammation , such as redness of the peritoneal surface and the presence of cloudy exudate. The talc had spread diffusely through the peritoneal cavity.

Rabbits 3, 4, and 5 had faulty healing of the peritoneal incision, so that during the experiment some AFIF solution leaked through a peritoneal fistula.

To determine the nature of the peritoneal exudate in the experimental animals a sample of the exudate was examined bacteriologically and histologically on three different occasions. None of the samples showed any growth when cultured in nutrient broth, nor could any bacteria be found on direct smears. In smears stained with Wright's stain (26) followed by Giemsa stain (26) the exudate was seen to contain large numbers of neutrophils, fewer monocytes, and some red cells.

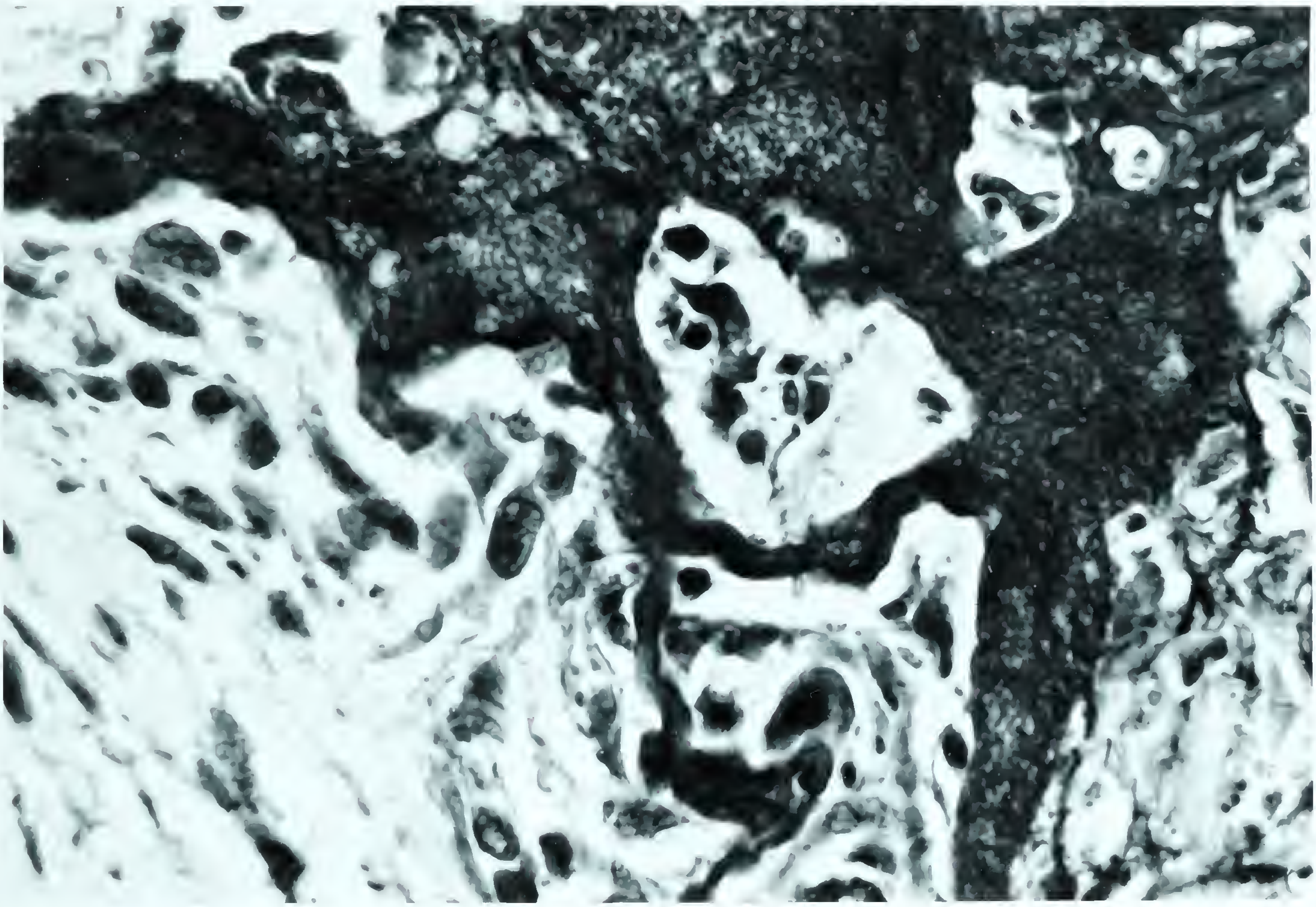


Low power photomicrograph of a section through an adhesion between intestines, stained with phosphotungstic acid.

1 and 1' are degenerating serosal surfaces.

2 is fibrin.

Between the serosa and fibrin are areas of fibroblast proliferation and collagen formation. The fibroblasts appear to be invading the central area.



High power photomicrograph of the area outlined on the previous page.

The dark area is fibrin, which is being invaded by fibroblasts.

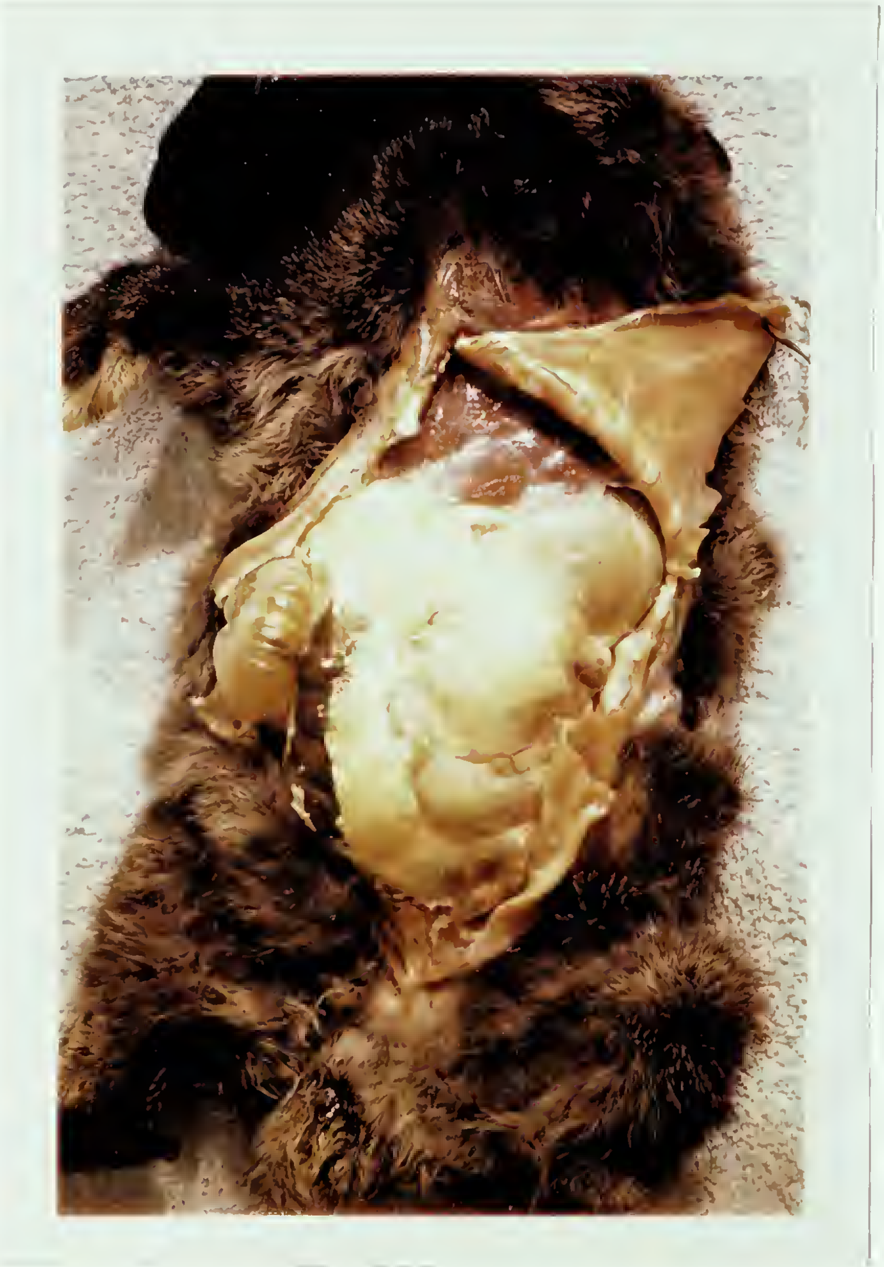


Figure 5

The effect of intraperitoneal injections of fibrinogen on adhesion formation.

Procedure:

Two rabbits were prepared surgically as for the previous experiments. Half an hour after the operation the animals were injected with 30 ml of sterile fibrinogen in saline solution (19 mg/ml). After three hours each animal received 3 grams of talc in a sterile suspension intraperitoneally. After four hours the rabbits received another 30 ml of the same fibrinogen solution, this being the first of a series of similar injections given every eight hours for five days. At the end of this period the same examinations were done as in the above experiments.

Results:

The gross appearance of the peritoneal cavity is shown in figure 5. The injected fibrinogen had clotted so that the peritoneal viscera were seen embedded in one large mass of fibrin.

The haematological data are given below.

Table 3

Rabbit	W.B.C.	PL.	Ht.	Hb.	Fb.	Th time secs.
1	5280	840000	35	11	690 640	12
2	4640	750000	34	10.2	790 665	11

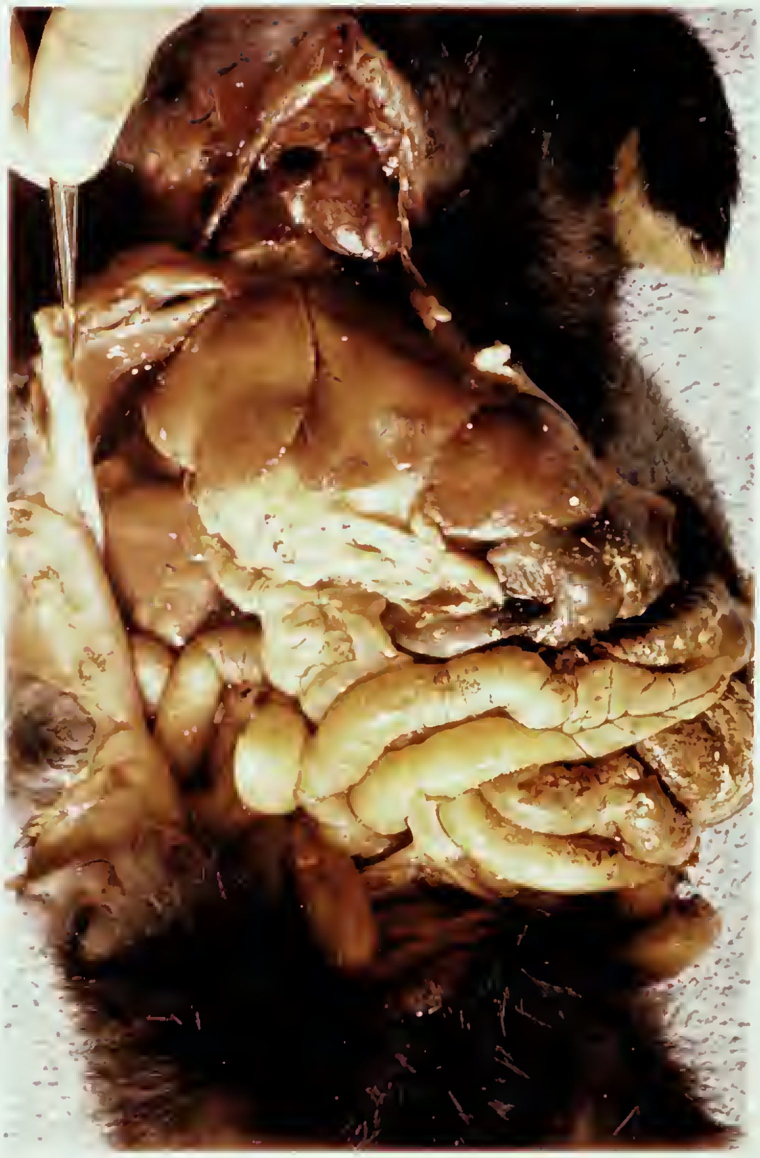


Figure 6

The effect of intraperitoneal heparin injections with the presence of talc in the peritoneal cavity.

Procedure:

Three rabbits were prepared surgically as before. One hour after the operation a dose of 15 mg per kg body weight of heparin was administered intraperitoneally in the form of a sterile saline solution containing 2 mg heparin per ml. Two hours there after 3 grams of talc were injected into the peritoneal catheter. After another hour had elapsed the rabbit was placed in a rabbit box and the catheter hooked up to a 50 ml syringe in the continuous flow apparatus, which delivered 24 ml of the heparin solution over a period of 6 hours.

Results:

Two rabbits were found dead the following morning, while the third rabbit survived a day and a half. The appearance of the peritoneal cavity is shown in figure 6. In all three cases the peritoneal cavity was found full of blood, and the talc was distributed diffusely through the cavity. The animals had an exsanguinated appearance, and death was thus likely due to peritoneal haemorrhage caused by irritation of the talcum powder and the absence of thrombin. One rabbit showed intrapulmonary haemorrhage.

Part 2. EFFECT OF PARENTERAL AFIF AND ALBUMIN ON FIBRINOGEN CONCENTRATIONS

Introduction:

Although the six hourly doses administered to the rabbits used for adhesion studies were mostly larger than the doses given to the rabbits for adsorption studies, the blood of the former group clotted firmly at the end of the five day experiment. This observation suggested that either the ability of the peritoneum to absorb AFIF had decreased and / or the anticoagulant activity of the AFIF solutionfor the particular animal had decreased. The last conclusion if true, would greatly hinder studies of the effect of AFIF on connective tissue formation other than by local application.

During the following experiments the above observation was confirmed and its nature made clear.

Anticoagulant activity of intraperitoneal AFIF at the beginning and end of a five day period of AFIF administration.

Procedure:

A rabbit was prepared with a peritoneal catheter as before . An intraperitoneal dose of 750 mg AFIF per kg body weight was administered. Three and a half hours

after this injection, a blood sample was taken by heart puncture for a Lee-White clotting time (25) and a thrombin time determination. Eight hours after the first injection a same dose of AFIF was given and this was repeated every eight hours for five days. Three and a half hours after the last injection another blood sample was taken for clotting time and thrombin time determinations.

Results:

First sample-	clotting time	3.5	minutes
	thrombin time	62	seconds
Second sample-	clotting time	4	minutes
	thrombin time	18	seconds

On autopsy a large amount of clear viscous fluid resembling AFIF was found in the peritoneal cavity.

An attempt to maintain in vivo incoagulability of rabbit blood by repeated intravenous injections of AFIF.

Procedure:

A jugular catheter was inserted in each of two rabbits and the end of the catheter was made to protrude through a small incision on top of the skull. The incision in the neck was then closed by simple interrupted sutures and the animal allowed to recover from the anaesthetic. A dose of 500 mg AFIF per kg body weight was

injected into the jugular catheter. Five minutes later a couple ml of blood were run from the catheter and discarded, and then a one ml blood sample was taken to test its coagulability. Every hour there after for seven hours a sample was withdrawn and time allowed for clotting. After seven hours the rabbits were placed in a rabbit box and given a continuous infusion at the rate of 500 mg AFIF per six hours. The following day the coagulability of the blood was again tested.

Results:

The first dose of AFIF rendered the blood of one rabbit incoagulable for five hours and the blood of the second rabbit for six hours. After one day the same dose of AFIF did not make the blood incoagulable any more.

There was no bleeding from the surgical incision nor any haematoma formation.

The effect of a single dose of AFIF on AFIF titration, thrombin time, and the plasma fibrinogen level.

Procedure:

Nine rabbits were used for this experiment. Thirteen mls of blood were taken by cardiac puncture, nine of which were used to titrate with an AFIF solution and the remaining four were oxalated. The plasma was collected from the four ml of blood. One ml of plasma was used to determine the fibrinogen level and the remainder frozen and stored till the next day. The fibrinogen determination was not done in duplicate in order to take as little blood from the rabbit as possible, thus reducing possible effects caused by blood loss. The titration value obtained above was used to calculate the amount of AFIF necessary to render the rabbits blood incoagulable. A dose slightly in excess of this was administered intravenously into the marginal ear vein over a period of five minutes. After 24 hours a larger blood sample than before was taken and a second AFIF titration and fibrinogen determination (this time in duplicate) were done. The remaining plasma was mixed with AFIF in the proportions indicated in table 4, and the thrombin time of the mixture determined. The frozen plasma from the previous day was thawed out, mixed with the same AFIF in the same

proportions and a thrombin time was done using the same thrombin solution.

Results:

Effect of I.V. AFIF

Table 4

No.	Dose mg/kg	Titr. 1	Titr. 2	Fb. 1	Fb. 2	Th. t.1 seconds	Th. t. 2 seconds
1	390	1/6	1/2			9	13
2	410	1/4	1/1	270	430	30	19.5
3	410	1/8	1/7	290	412	∞	55
4	400	1/8	1/8	270	370	30	59
5	400	1/8	1/5	294	370	20	56
6	320	1/10	1/6	247	295	56	30
7	330	1/8	1/6	295	290	30	26
8	390	1/10	1/7	200	355	30	30
9	380	1/9	1/4	267	475	26	26

Titr. is the lowest value of AFIF solution/blood which remained incoagulable.

Thrombin times of No. 1 were done on whole blood on two separate days. The other thrombin times were done as described in the procedure. In No. 2 the thrombin time was done on a mixture of 1 part AFIF and 8 parts of blood; in No. 3 on a mixture of 1 part AFIF and 10 parts of blood; in 4 and 5 on mixtures of .8 parts AFIF and 10 parts of blood; and the rest on mixtures of .7 parts AFIF and 10 parts of blood.

The effect of raising plasma fibrinogen in vitro on AFIF titration.

In the previous experiments it became obvious, that with the decrease of anticoagulant activity of AFIF there was a simultaneous rise in the fibrinogen concentration. This experiment was done with the object to demonstrate a relationship between fibrinogen concentration and AFIF activity.

Procedure:

Fresh blood from five different rabbits was mixed in various proportions with AFIF in saline solution. In one such series of mixtures a small amount of fibrinogen in buffered saline solution had been added previously to the AFIF solution, while in a second and identical series of mixtures, serving as controls, buffered saline had been added. (The buffer was 1/10 volume 0.2 M imidazole buffer of pH 7.52). The content of each tube is given in tables 5 and 6. After addition of the blood the tubes were covered with Parafilm, their content mixed by inverting three times and then placed in a water bath at 37°C. After half an hour the tubes were examined for the presence of coagulation. The plasma fibrinogen concentrations were determined for all rabbits.

Results: Effects of increased fibrinogen concentration

Table 5

Tube No.	No.'	AFIF	Fb. in No. Saline in No.'	Blood	Clotting in No.	Clotting in No.'
1	1'	0.17	0.23	1.60	yes	yes
2	2'	0.20	0.23	1.57	yes	no
3	3'	0.22	0.23	1.55	yes	no
4	4'	0.25	0.23	1.52	yes	no
5	5'	0.29	0.23	1.48	yes	no
6	6'	0.33	0.23	1.44	no	no
7	7'	0.40	0.23	1.37	no	no

All above values are in mls. AFIF concentration is 37.5 mg/ml. Fibrinogen concentration is 9 mg/ml. Blood is from the first rabbit.

Table 6

Tube No.	No.'	AFIF	Fb. in No. Saline inNo.'	Blood	Clotting in No.				Clottingin No			
					#2	#3	#4	#5	#2	#3	#4	#5
1	1'	0.18	0.15	1.67	yes	yes	yes	yes	no	yes	yes	no
2	2'	0.20	0.15	1.65	yes	yes	yes	yes	no	no	no	no
3	3'	0.22	0.15	1.63	yes	yes	yes	yes	no	no	no	no
4	4'	0.25	0.15	1.60	no	yes	yes	yes	no	no	no	no
5	5'	0.29	0.15	1.56	no	no	no	no	no	no	no	no
6	6'	0.33	0.15	1.52	no	no	no	no	no	no	no	no
7	7'	0.40	0.15	1.45	no	no	no	no	no	no	no	no

Fb. conc. is 13.4 mg/ml. # means rabbit. Other values as above.

From tables 5 and 6 it can be seen that the increased fibrinogen concentration increased the minimum amount of AFIF necessary to prevent clotting.

The plasma fibrinogen concentrations of the five rabbit blood samples were as follows:

Rabbit	Fb. concentration
#1	270 mg/100cc plasma
#2	278 mg/100cc plasma
#3	278 mg/100cc plasma
#4	311 mg/100cc plasma
#5	288 mg/100cc plasma

The effect of a single dose of albumin on AFIF titration and the plasma fibrinogen level.

Procedure;

Five rabbits were used for this experiment. A blood sample was taken for AFIF titration and fibrinogen determination. Then 370 mg albumin/kg. body weight were injected intravenously. The albumin was dissolved in a saline solution(40 mg./cc.). After 24 hours another blood sample was taken and the same determinations were repeated using the same AFIF solution for titration as before.

Results: Effects of I.V. Albumin

Table 7

Rabbit	Titr. 1	Titr. 2	Fibr. 1	Fibr. 2
1.	1/1	1/1	320	400
2.	1/3	1/2	264	420
3.	1/8	1/5	295	420
4.	1/8	1/5	310	440
5.	1/9	1/6	225	460

Values of units as before.

The effect of single saline injections on the AFIF titrations and the plasma fibrinogen levels.

Procedure:

Exactly the same experimental procedures were used as above, with the only difference that normal saline was used in the place of albumin. The dose of saline was 9.4 ml./kg. body weight, or the same volume as used for the albumin injections.

Results:

Effects of I.V. Saline

Table 8

Rabbit	Titr. 1	Titr. 2	Fibr. 1	Fibr. 2
#1	1/8	1/6	278	325
#2	1/9	1/9	230	245
#3	1/8	1/8	300	300
#4	1/9	1/9	208	194
#5	1/9	1/8	225	325
#6	1/9	1/9	295	330
#7	1/9	1/9	290	278

Statistical evaluation of the rise in fibrinogen levels:

A T-test was done on the fibrinogen values before and after the injection of AFIF, albumin and saline respectively.

The probability that the results were not signif-

icant wasless than 1% for the experiment with AFIF. For the albumin experiment the probability was less than 1%. The increase when saline was injected was not significant at the 5% point.

DISCUSSION

From the results of the first part of this thesis it is obvious that AFIF did interfere with the formation of adhesions. To realize the nature of this interference the action of AFIF should be considered first.

AFIF is an anticoagulant which interferes mainly with the last step of coagulation, where fibrinogen is converted into fibrin through the catalytic action of thrombin. It also inhibits thromboplastin formation to a lesser extent.(21,27,28,29,30) The nature of the inhibitionof the conversion of fibrinogen to fibrin is not completely known and remains for the present a controversial issue. Triantaphyllopoulos and Kowalski and co-workers produced evidence suggesting that AFIF competes both with fibrinogen for thrombin, and retards the polymerization of the fibrin monomer. Alkjaersig and Fletcher on the other hand, maintain that the action of AFIF is solely due to interference with the polymerization of the fibrin monomers.(31) For the work presented here the important point is that most workers agree that AFIF interferes mainly with the last step in blood coagulation and that AFIF from bovine origin does not prevent the consumption of prothrombin. (32)

Heparin acts both in the earliest phase of clotting and together with anti-thrombin 11 as an anti-thrombin.(33)

We can therefore assume that the reason for the presence of severe intra-abdominal bleeding in the rabbits receiving heparin,and the absence of such bleeding in the rabbits receiving AFIF was due mainly to the absence of a physiologically active thrombin in the former and its presence in the latter. There is evidence that small amounts of thrombin will cause the platelets to stick and undergo viscous metamorphosis.(34) Thrombin also releases the vasoconstrictor serotonin from platelets .(35) It is likely that the two above mechanisms accounted for the effective hemostasis in the AFIF treated rabbits, whereas in the heparinized rabbits platelet clumping does not take place. It is less likely that the difference was due to the fact that the AFIF acted only locally during the five day period, because in at least one of the rabbits, complete incoaguability of blood taken from the ear vein was apparent a couple of hours after the first injection of AFIF. In addition, Triantaphyllopoulos (32) observed effective hemostasis in rabbits whose blood was rendered completely incoaguable by intravenous AFIF infusions and similar observations were made in this thesis.

It is interesting to observe that the inflammation produced by the talc in the peritoneum of heparinized rabbits was severe enough to damage the small blood vessels underlying the peritoneal mesothelium sufficiently to cause extensive bleeding. This demonstrates the potential danger of inflammatory reaction in hemophylic patients and patients suffering from other hemorrhagic disorders caused by a lack of thrombin formation.

From histological and gross examinations, and taking into account the work of Menkin, the following steps can be postulated in the formation of peritoneal adhesions: irritation of the peritoneal surface; production of an inflammatory exudate; clotting of the exudate, with the subsequent sticking together of the two apposing peritoneal surfaces; loss of mesothelial definition and invasion of the fibrin by fibroblasts, accompanied by the laying down of callagen fibers and the invasion of capillary buds.

Since the prevention of adhesion formation by AFIF was shown to be the result of its inhibition of fibrin formation, the conclusion may be drawn, that the laying down of fibrin is an early and essential step in the formation of the connective tissue of adhesions. Spagna and Peskin (36) had shown essentially the same

by preventing the adhesions which normally follow the crushing of the cecum of a rat by intraperitoneal fibrinolysin injections. However since the trauma was minor and temporary the gross appearance of the adhesions in their control rats was loose and fibrinous, and no histological studies of these adhesions were done.

In three of the rabbits treated with AFIF, adhesion formation had taken place between the liver and the diaphragm. A possible explanation for this may be that the increased volume of the peritoneal cavity caused by the large volumes of fluid injected caused a relatively close apposition of the peritoneal surfaces of the liver and diaphragm, reducing the volume of AFIF bathing these surfaces. It can thus be reasoned that the inflammatory exudate in this area diluted the small amount of AFIF to such an extent that it could not prevent fibrin formation.

The experiment in addition demonstrated the localizing and protective actions of fibrin. In the control animals the peritoneal surface had a shiny healthy appearance and the talc was localized in a few large lumps, in contrast with the peritoneum of the experimental animals which looked inflamed (whitish exudate and redness of the peritoneal surface), and talc crystals

were dispersed throughout the peritoneal cavity.

It should be noted that the leukocyte count was in the normal range for both the experimental and control animals. The haematocrit and haemoglobin were found to be depressed to the same extend in both groups, which may at least in part be due to haemodilution. AFIF did not affect the platelet count. The increased thrombin times in the AFIF treated rabbits indicate the presence of AFIF in the blood stream. Another indication of the presence of AFIF in the blood is that the difference between the fibrinogen determinations in saline and in distilled water seen in the AFIF treated rabbits was greater than in the albumin treated rabbits. Triantaphyllopoulos (31) showed this to be due to the fact that AFIF is active in saline, but not in distilled water.

The control animals injected with fibrinogen showed the opposite effect of the AFIF injections, namely one large fibrin mass connecting all the peritoneal viscera together.

The large rise in the plasma fibrinogen levels obser~~e~~ved in the adhesion experiments was not surprising . Foster and Whipple (37) as early as 1922, reported on the effects of a number of experimental conditions and diseases on the plasma fibrinogen levels. They concluded

that diseases and conditions, which stimulate or depress liver function, in a like manner and in equal degree, stimulate or depress fibrinogen production. Since then a rise in fibrinogen concentration has been reported in a large number of clinical conditions. Elliot and Buckell (38) in a short survey listed the following: typhoid fever, bronchopneumonia, rheumatic arthritis, leukemia, coronary thrombosis, arteriosclerosis, ankylosing spondylitis, cardiac infarction, multiple sclerosis, and chronic epileptic encephalitis. Fensdorff et al. (39) reported on extremely high fibrinogen levels in famillial mediterranean fever. Elliot and Buckel, and Losner et al. found a rise in fibrinogen concentration to be of prognostic value in cerebral accidents and myocardial infarctions. Both groups found the prognosis to be grave if after an attack the fibrinogen level rose to over 800 mg per 100 ml of plasma. This led Losner and Volk (40, 41) to believe that the extend of the rise in fibrinogen concentration reflected the amount of tissue dammage. When this group experimentally produced infarctions of various sizes in a number of dogs, they indeed found the largest rise in fibrinogen to be associated with the most extensive infarctions (42).

Hidalgo et al. studied the effect of tissue

dammage in rabbits on their fibrinogen levels. They found a fall in fibrinogen immediately after extensive tissue dammage, followed after 24 hours by a rise. They postulated that the initial fall was caused by a release of tissue thromboplastin with subsequent thrombin formation and intravascular clotting of fibrinogen, and that the subsequent rise in fibrinogen was due to the stimulating effect of thromboplastin on the liver. It seems doubtfull that thromboplastin should be the factor responsible for the rise in fibrinogen concentration. Foster and Whipple stimulated increased fibrinogen concentrations in dogs by feeding high protein diets, and in this thesis a rise in fibrinogen was obtained by a single intravenous injection of foreign proteins. In these two latter experimental conditions there was no significant release of tissue thromboplastin. It seems thus logical to agree with Foster's and Whipple'sconclusion that the elevation of the plasma fibrinogen level is a non specific response to any condition which will stimulate liver function.

It is worth noting that although the rise in fibrinogen concentration is non specific, it may be an effective mechanism to correct potential haemorrhagic conditions due to the presence of circulating AFIF.

Simultaneous with the rise in fibrinogen there was a generally corresponding increase in the minimum concentration of AFIF necessary to render the blood incoagulable. Numbers 4 and 7 of table 4 constitute the only discrepancies to this statement, and may be due to unavoidable experimental errors. When the fibrinogen concentration was increased in vitro to the same extend as was observed in vivo, there was a similar decrease in AFIF activity. It can thus be concluded that the inability to maintain the blood of a rabbit incoagulable for an extended period of time by multiple AFIF injections of equal dosage was due to an increase in the plasma fibrinogen concentration.

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